

## Mutation in GNE is associated with a Severe Form of Congenital Thrombocytopenia

Futterer, Jane; Dalby, Amanda; Lowe, Gillian; Johnson, Ben; Simpson, Michael; Motwani, Jayashree; Williams, Mike; Watson, Steve; Morgan, Neil

DOI:

[10.1182/blood-2018-04-847798](https://doi.org/10.1182/blood-2018-04-847798)

License:

None: All rights reserved

*Document Version*

Peer reviewed version

*Citation for published version (Harvard):*

Futterer, J, Dalby, A, Lowe, G, Johnson, B, Simpson, M, Motwani, J, Williams, M, Watson, S & Morgan, N 2018, 'Mutation in GNE is associated with a Severe Form of Congenital Thrombocytopenia', *Blood*.  
<https://doi.org/10.1182/blood-2018-04-847798>

[Link to publication on Research at Birmingham portal](#)

### **Publisher Rights Statement:**

This research was originally published in Blood Online. Jane Futterer, Amanda Dalby, Gillian C. Lowe, Ben Johnson, Michael A. Simpson, Jayashree Motwani, Mike Williams, Steve P. Watson and Neil V. Morgan on behalf of the UK GAPP Study Group. Mutation in GNE is associated with a Severe Form of Congenital Thrombocytopenia. *Blood*. Prepublished 06/2018, <https://doi.org/10.1182/blood-2018-04-847798>

### **General rights**

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

### **Take down policy**

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact [UBIRA@lists.bham.ac.uk](mailto:UBIRA@lists.bham.ac.uk) providing details and we will remove access to the work immediately and investigate.

# **MUTATION IN *GNE* IS ASSOCIATED WITH A SEVERE FORM OF CONGENITAL THROMBOCYTOPENIA**

Jane Futterer<sup>1</sup>, Amanda Dalby<sup>1</sup>, Gillian C. Lowe<sup>1</sup>, Ben Johnson<sup>1</sup>, Michael A. Simpson<sup>2</sup>, Jayashree Motwani<sup>3</sup>, Mike Williams<sup>3</sup>, Steve P. Watson<sup>1</sup> and Neil V. Morgan<sup>1</sup> on behalf of the UK GAPP Study Group

<sup>1</sup>Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham

<sup>2</sup>Division of Genetics and Molecular Medicine, King's College, London, United Kingdom

<sup>3</sup>Department of Haematology, Birmingham Children's Hospital, Birmingham

Correspondence to:

Dr Neil V. Morgan, Institute of Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom.

Tel: (+44) 121 414 6820      Fax: (+44) 121 415 8817

n.v.morgan@bham.ac.uk

Inherited thrombocytopenias are associated with bleeding of all types of severity depending on the reduction in platelet count and whether there is altered platelet function.<sup>1</sup> The normal range for platelet counts varies by up to threefold, but an individual's platelet count is normally maintained within a narrow range. This requires a constant balance between thrombopoiesis, and platelet senescence and consumption. Heritable forms of thrombocytopenia are frequently caused by genes which regulate megakaryocytic differentiation and/or platelet production. Next generation sequencing strategies, such as whole exome sequencing, are efficient in identifying gene mutations that cause Mendelian disorders.<sup>2-4</sup> In this study, we used a whole exome-sequencing approach, to elucidate the genetic basis of a severe form of congenital thrombocytopenia.

We present a UK consanguineous family of Pakistani origin with two cousins with severe thrombocytopenia (Figure 1B). The proband, III:5, was aged three years with a platelet count of  $3 \times 10^9/l$  when entered into the Genotyping and Phenotyping of Platelets (GAPP) study. He was born by emergency caesarean section at 34 weeks gestation and had neurological symptoms shortly after birth and bilateral intraventricular haemorrhages. He had a ventriculo-peritoneal shunt inserted which required several revisions, with HLA platelet transfusion prophylaxis. He has developmental delay, skull abnormalities secondary to hydrocephalus and nystagmus. His baseline platelet count has remained at approximately  $10 \times 10^9/l$ . He received HLA matched platelet transfusions every 1-2 weeks for the first twelve months of life and his platelet count incremented well hereafter. There are no other abnormalities in the blood count. The bone marrow aspirate and trephine showed a normocellular specimen with normal megakaryocyte numbers and morphology and normal cytogenetics. Patient III:3 was aged seven years when recruited into the study. She has a baseline platelet count of  $15-20 \times 10^9/l$  and receives weekly HLA matched platelet transfusions to minimize symptoms from epistaxis and haematomata, previously causing hospitalisation. In both patients, coagulation parameters were normal and there were no anti-platelet autoantibodies or HLA antibodies. Blood (15 ml) from patients and healthy controls was taken in 10% by volume 3.8% trisodium citrate. Platelet rich plasma (PRP) was prepared and flow cytometry was conducted as previously described.<sup>5</sup> Transmission electron microscopy was performed as described<sup>6</sup> and examined using a JEOL 1200EX transmission electron microscope (Hertfordshire, UK). The number of  $\alpha$ -granules per  $\mu m^2$  was calculated for at least 40 platelets from each patient/control. The whole exome of the two affected individuals was sequenced with the SureSelect human All Exon 50Mb kit (Agilent Technologies) and sequencing on the HiSeq 2000 (Illumina) with 100 bp paired-end reads. The sequences were aligned to the reference genome (hg19 build).<sup>5</sup> To verify candidate

mutations, Sanger sequencing was performed using standard methods on an ABI 3730 automated sequencer.

Both patients have severe thrombocytopenia with platelet counts in platelet rich plasma (PRP) of :- patient III:5  $1.5 \times 10^7/\text{ml}$  and patient III:3  $2.5 \times 10^7/\text{ml}$ , reference range  $2.1\text{--}7.1 \times 10^8/\text{ml}$  [mean $\pm$ 2s.d.]. Mean platelet volume in patient III:5 and III:3 was 15.0fl and 10.4fl respectively (reference range 7.68-10.0fl). Parental platelet counts were normal. An extremely high Immature Platelet Fraction (IPF) of 87 and 83% (normal range 1.3-10.8%, n=40) was found in patients III:5 and III:3 respectively which suggests rapid production (possibly due to rapid clearance). Flow cytometry was used to assess platelet function in the two affected individuals, using an assay validated for activated platelets using dilutions of PRP from healthy volunteers. The levels of surface glycoproteins CD42b (GPIb $\alpha$ ), CD41 ( $\alpha$ IIb) and GPVI in patient III:3 were within the reference ranges established in healthy volunteers, whereas for patient III:5, the levels of CD42b and CD41 fell outside. This could suggest global platelet dysfunction, where loss of glycans can lead to failure of the receptor being transported to the surface, or to increased proteolysis (Figure 2A). Patient III:5 also showed a complete abolition of CD62P (P-selectin) expression and a very weak increase in binding of fibrinogen to ADP, CRP and PAR-1 (Figure 2B and 2C). Patient III:3 showed a slightly greater increase in fibrinogen binding than III:5 to most agonists and a recovery of CD62P expression to high concentrations of CRP and PAR1 peptide, although this was below the range of responses to healthy controls in all cases. Electron microscopy of patient platelets revealed that these are enlarged but with a similar number of  $\alpha$ -granules per surface area, compared to controls (Figure 2D).

The exome of both patients was sequenced and the alignment of the sequencing reads revealed 23,943 and 24,293 variations in patients III:3 and III:5 respectively. Comparisons within the EVS, 1000G and our in house GAPP database of over 1200 exomes identified two homozygous non-synonymous variants and 1 non-frameshift deletion that were present in both patients (Supplementary Tables 1 and 2), with all variations mapping to a tightly-linked homozygous region on chromosome 9p13.3 (Supplementary Table 2). The two non-synonymous variants were in genes *GNE* (p.G416R) and *FRMPD1* (p.A509V) and the non-frameshift deletion in *ANKRD18A* (p.Glu801del). Family studies using Sanger sequencing confirmed that all three variants segregated with disease status (Figure 1B). Pathogenicity was predicted using four separate *in silico* based pathogenicity prediction softwares (MutationTaster, SIFT, PROVEAN and PolyPhen-2) and conservation at the site of variation was determined by PhyloP and PhastCons. Together all three variants were classified as “unknown significance” when considering the ACMG consensus guidelines. Upon further

analysis within the ExAC database, only the variants within *ANKRD18A* and *GNE* were novel. Data from RNA sequencing of hematopoietic progenitors (blueprint.haem.cam.ac.uk), suggested that there was very low expression of *ANKRD18A* mRNA in hematopoietic progenitors. This is in contrast to *GNE* mRNA, which is expressed widely in hematopoietic progenitors.

In previous studies, two compound heterozygous variations in the gene encoding *GNE* have been noted to cause a disorder of progressive muscle weakness with a secondary symptom of thrombocytopenia.<sup>7 8</sup> Previous dominant mutations in *GNE* have been associated with sialuria.<sup>9 10</sup> It is important to note that the recessive patients presented with severe body myopathy as a primary symptom, while the patients in our study do not display signs of myopathy although this is possibly because of their age. Furthermore a previous study involving the whole exome sequencing of a single pedigree with severe thrombocytopenia and bleeding identified an apparent *PRKACG* variant but a strong candidate variant in this family was also a homozygous missense variant in the kinase domain of *GNE* (p.G559R) as shown in Figure 1C.<sup>11</sup>

*GNE* encodes Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-acetylmannosamine kinase, a bi-functional enzyme involved in the sialic acid biosynthesis pathway and is expressed within all cells of the haematopoietic lineage. Thrombocytopenia is known to be associated with increased platelet desialylation in septic patients due to altered platelet production/survival.<sup>12</sup> Further, platelet counts are increased in a cohort of influenza patients treated with the sialidase inhibitor, oseltamivir (Tamiflu).<sup>13</sup> A platelet clearance system has been shown to exist for desialylated platelets involving macrophages and hepatocytes.<sup>14</sup> The Ashwell-Morell receptor binds platelets with reduced sialic acid expression<sup>15</sup> and removal of just 8-10% of sialic acid residues by neuraminidase treatment leads to increased platelet clearance rates *in vivo*.<sup>16</sup>

In summary, our results indicate that the *GNE* mutation described here leads to macrothrombocytopenia, possibly due to a reduction in sialic acid biosynthesis, which is expected to cause increased removal of platelets and altered platelet formation.

**Acknowledgements**

We thank the families for providing samples and our clinical and laboratory colleagues for their help. This work was supported by the British Heart Foundation (PG/13/36/30275; FS/13/70/30521; RG/09/007), an MRC Doctoral Training Partnership grant (BJ), a Wellcome Trust Combined Training Programme Fellowship (093994) (GCL). We thank the NIHR Haematology Specialty Group for their help in recruiting to the study, and all our clinical investigators and collaborators. The authors acknowledge support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust.

**Authorship Contributions**

GCL, SPW and NVM designed the research. JM and MW provided patient samples and clinical data. GCL and NVM undertook the research governance of the study. JF, AD, GCL, BJ, MS and NVM performed the research and analyzed data. NVM and AD wrote the paper and all authors critically reviewed and edited the paper.

**Disclosure of Conflicts of Interest**

The authors declare no competing conflicts of interest.

## References

1. Nurden AT and Nurden P. Inherited thrombocytopenias. *Haematologica* 2007;92(9):1158-64.
2. Jones S, Hruban RH, Kamiyama M, et al. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* 2009;324(5924):217.
3. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 2009;361(11):1058-66.
4. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* 2009;461(7261):272-6.
5. Johnson B, Lowe GC, Futterer J, et al. Whole exome sequencing identifies genetic variants in inherited thrombocytopenia with secondary qualitative function defects. *Haematologica* 2016;101(10):1170-1179.
6. Fletcher SJ, Johnson B, Lowe GC, et al. SLFN14 mutations underlie thrombocytopenia with excessive bleeding and platelet secretion defects. *J Clin Invest* 2015;125(9):3600-5.
7. Izumi R, Niihori T, Suzuki N, et al. GNE myopathy associated with congenital thrombocytopenia: a report of two siblings. *Neuromuscul Disord* 2014;24(12):1068-72.
8. Zhen C, Guo F, Fang X, Liu Y and Wang X. A family with distal myopathy with rimmed vacuoles associated with thrombocytopenia. *Neurol Sci* 2014;35(9):1479-81.
9. Eisenberg I, Avidan N, Potikha T, et al. The UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. *Nat Genet* 2001;29(1):83-7.
10. Seppala R, Lehto VP and Gahl WA. Mutations in the human UDP-N-acetylglucosamine 2-epimerase gene define the disease sialuria and the allosteric site of the enzyme. *Am J Hum Genet* 1999;64(6):1563-9.
11. Manchev VT, Hilpert M, Berrou E, et al. A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene. *Blood* 2014;124(16):2554-63.
12. Li MF, Li XL, Fan KL, et al. Platelet desialylation is a novel mechanism and a therapeutic target in thrombocytopenia during sepsis: an open-label, multicenter, randomized controlled trial. *J Hematol Oncol* 2017;10(1):104.
13. Jansen AJ, Peng J, Zhao HG, Hou M and Ni H. Sialidase inhibition to increase platelet counts: A new treatment option for thrombocytopenia. *Am J Hematol* 2015;90(5):E94-5.
14. Sorensen AL, Rumjantseva V, Nayeb-Hashemi S, et al. Role of sialic acid for platelet life span: exposure of beta-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood* 2009;114(8):1645-54.
15. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat Med* 2015;21(1):47-54.
16. Greenberg J, Packham MA, Cazenave JP, Reimers HJ and Mustard JF. Effects on platelet function of removal of platelet sialic acid by neuraminidase. *Lab Invest* 1975;32(4):476-84.

## Figures

**Figure 1. Identification of a homozygous missense substitution in *GNE*.** (A) Filtering strategy of whole exome sequencing results to identify candidate variants in patients III:3 and III:5 of the same family. (B) Segregation analysis of the exome candidates in family members where DNA was available. The three variants (in the genes *GNE*, *FRMPD1* and *ANKRD18A*) were shared by both children and were located within a region of homozygosity on chromosome 9p13.3. Double lines linking parents signify first cousin unions. (C) Linear domain organisation of *GNE* encoding the enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase. Experimental allosteric sites are based on *in vitro* studies (AS), region of unknown function (UF). The approximate position of amino acid substitutions (p.G416R and p.G559R) found in the family in this study and in an independent study<sup>11</sup> respectively are indicated and based on transcript NM\_005476.

**Figure 2. Flow cytometry and transmission electron microscopy assessment of platelet function in patients III:3 and III:5.** Flow cytometry assessment of platelet function in patients III:3 and III:5 assessed on Accuri C6 flow cytometer. (A) Platelet glycoprotein receptors. (B) CD62P expression and (C) fluorescent fibrinogen binding following platelet stimulation by various agonists for 2 min. The platelet rich plasma from healthy controls was diluted 1 in 10 with phosphate buffered saline and served as a control range. Data for healthy volunteers shown as mean  $\pm$  1 s.d. (n= 9, except for GPVI where n=2)). (D) Transmission electron microscopy image of the platelets from patient III:5 and a healthy control platelets. Arrow indicates  $\alpha$  granule, graph shows number of alpha granules/surface area, scale bar = 2 $\mu$ m.

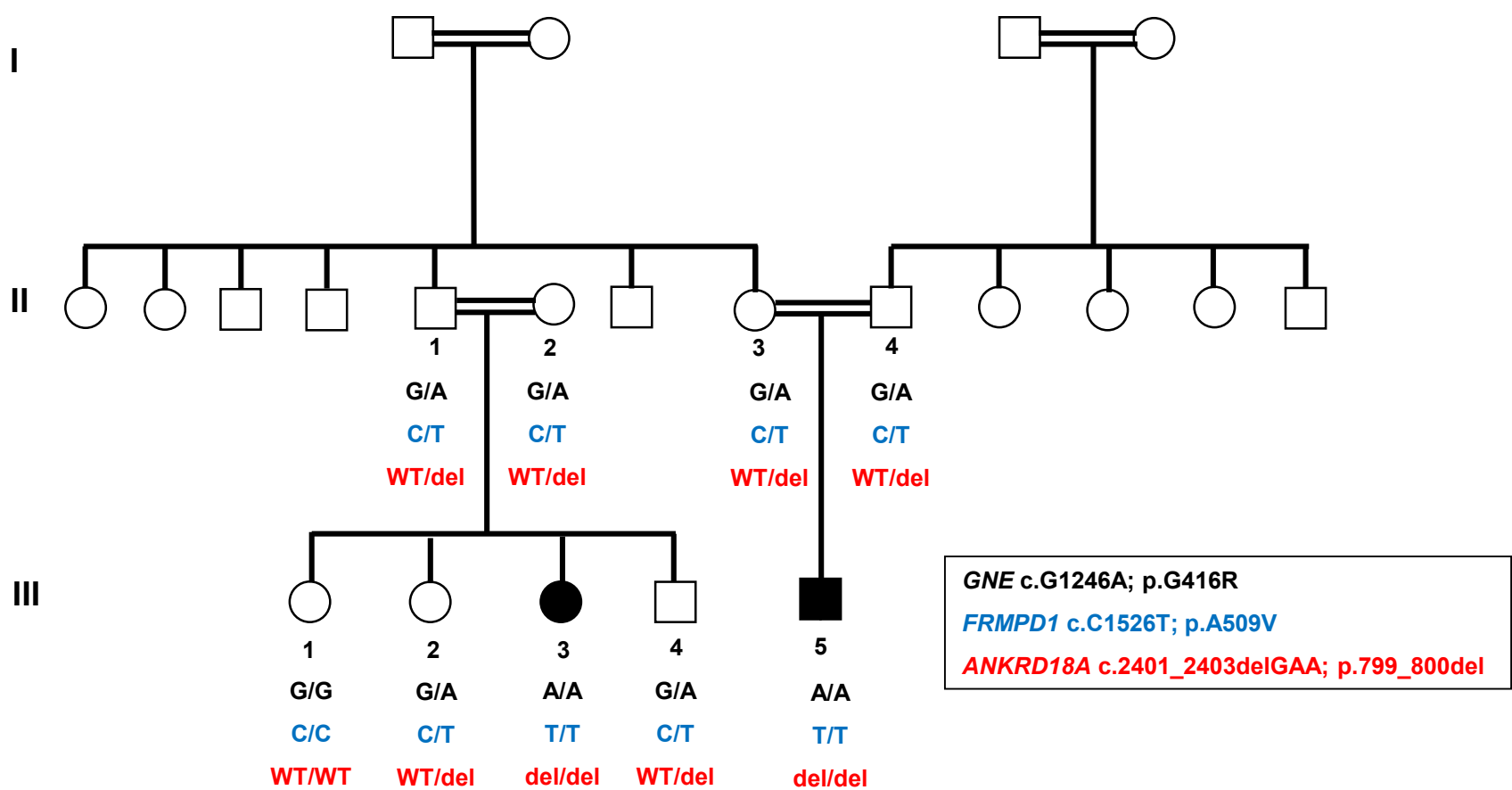


Figure 1

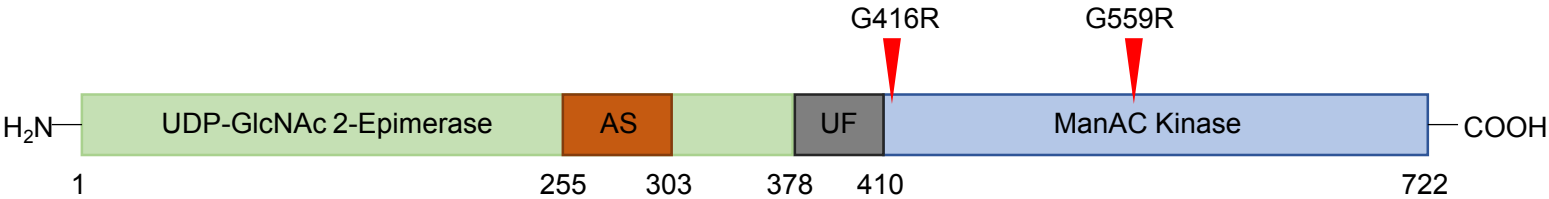
A

	Patient III:3	Patient III:5
Total variants	23,943	24,293
Novel or Rare variants	583	607
Homozygous	77	83
Overlapping variants	15	
In linkage regions	3	
Predicted damaging	2	

B



C



**Figure 2**